ISOLATION AND CHARACTERIZATION OF PEANUT (*Arachis hypogaea*) LECTIN AND STUDY OF ITS ANTI-CANCER PROPERTIES

Thesis submitted to **Department of life science for** ***the partial fulfillment Of the M.Sc. Degree in Life Science***

Department of Life science

NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA

राष्ट्रीय प्रौद्योगिकी संस्थान, राउरकेला

Submitted by

Md Khurshidul Hassan

MSc. Life Science, II year

Roll no. 411LS2047

Under the guidance of

Dr. Sujit Kumar Bhutia

Assistant Professor

NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA
राष्ट्रीय प्रौद्योगिकी संस्थान, राउरकेला

Dr. Sujit Kumar Bhutia
Assistant Professor.
Department of life sciences
National institute of Technology Rourkela
Rourkela-769008, Odisha, India
Ph-91-661-2462686
Email:sujitb@nitrkl.ac.in, bhutiaask@gmail.com

Ref. No.
Date:

CERTIFICATE

This is to certify that the thesis entitled "**Isolation and characterization of peanut (*Arachis hypogaea*) lectin and study of its anti-cancer properties"** which is being submitted by Mr. Md Khurshidul Hassan, Roll No. 411LS2047, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bona fide research work, carried out by him under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

Dr. Sujit K Bhutia

Dedicated to my
beloved parents and family

DECLARATION

I do hereby declare that the Project report entitled **"Isolation and characterization of peanut (*Arachis hypogaea*) lectin and study of its anti-cancer properties"** submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfillment of the Master Degree in Life Science is a faithful record of bona fide and original research work carried out by me under the guidance and supervision of **Dr. Sujit Kumar Bhutia**, Assistant Professor, Department of life Science, NIT, Rourkela.

Date: **Md Khurshidul Hassan**

Place:

ACKNOWLEDGEMENT

A research project like this is never the work of anyone alone. The influences of many different people, in their different ways, have made this possible. Before extending my sense of gratefulness towards those who were directly involved in the preparation of this thesis, I would like to extend my appreciation especially towards an awesome group of people who have always supported me in multiple ways throughout my life. I would like to acknowledge my parents for their time, affection, knowledge and experiences they shared with me.I convey my heartfelt affection to my sister and other family members for providing me with moral support and affection in times of need.

I express my deep sense of gratitude and reverence to my supervisor, Dr. Sujit Kumar Bhutia, Assistant Professor, Department of Life Science, NIT Rourkela, for his admirable guidance, constant and untiring supervision, help and encouragement throughout the investigation and preparation of this manuscript.

I am extremely grateful and indebted to Dr. S.K. Patra, HOD, Department of Life Science, NIT-Rourkela, Dr. Surajit Das, Dr. Bibekananda Mallick, Dr. Bismita Nayak, Dr. Rasu Jaybalan, and Dr. Suman Jha for their inspiring suggestions and valuable advice not only for this investigation but throughout my period of study at this institute.

I am highly obliged to Subhadip Mukhopadhyay, Prashanta Kumar Panda, Durgesh Nandini Das, Niharika Sinha, Ph.D Scholars, Department of Life Science, NIT-Rourkela, for their constant help, affection and encouragement during the period of this project. I learnt something from each one of them and this project would not have been possible without their guidance.

My heartfelt thanks to my friends Sandeep Dey, Bibhudutta Mishra, Himadri Tanaya Panda and all other classmates for their moral support, help and encouragement throughout the course of this work. I take this opportunity to acknowledge their constant help and support.

At the end, I bow down my head to the almighty whose omnipresence has always guided me. I thank God for the wisdom and perseverance that he has bestowed upon me during this research project, and indeed, throughout my life.

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LIST OF ABBREVIATIONS

%: Percentage

°C: Degree Celsius

ConA: Concanavalin A

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

EDTA: Ethylene Diamine Tetra Acetate

FBS: Fetal Bovine Serum

gm.: Gram

H: Hour

HA: Haemagglutination Assay

Kg: Kilogram

L: Liter

M: Molar

MEM: Minimum Essential Medium eagle

mg: Milligram

Min: Minute

mL: Milli Liter

mM: Milli molar

OD: Optical Density

PCD: Programmed Cell Death

penstrep :Penicillin-Streptomycin mixture

PNA: Pea Nut Agglutinin

SBL: SoyBean Lectin

SDS PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

µg: Micro gram

µm: Micro meter

1. ABSTRACT

Plant lectins are sugar binding proteins or glycoproteins of non-immune origin having the ability to agglutinate or precipitate glycoconjugates or both. In the present work, the isolation and characterization of the lectin from the legume plant peanut (*Arachis hypogaea*) has been done. The isolation of the lactose-binding lectin from the peanut seeds was carried out by performing affinity chromatography using a lactamyl sepharose column. Affinity chromatography elute was dialysed, followed by lyophilization and the isolated lectin was characterized by both SDS, native PAGE followed by haemagglutination assay with untreated and neuraminidase-treated human erythrocytes. Besides, the *in vitro* anti-cancer property of the isolated PNA was determined by performing cell viability assays viz. MTT assay and trypan blue exclusion assay using human cancer cell lines. Furthermore, the isolated lectin was found to induce DNA fragmentation by performing DNA laddering assay.DAPI staining was also done for determining its role in inducing DNA fragmentation in dose dependent manner.

Keywords: Peanut lectin, legume lectins, lactamyl sepharose, MTT assay, trypan blue assay, DAPI staining,clonogenic assay, DNA fragmentation, anti-cancer, autophagy, lectin-induced apoptosis, $IC_{50,}$ Peanut Agglutinin, affinity chromatography, SDS-PAGE, Haemagglutination.

2. INTRODUCTION

Since the turn of the 19th century, the existence in nature of certain proteins with activity to agglutinate erythrocytes, were subsequently shown to be sugar specific, and eventually named lectins, was well documented, but until about two decades ago they aroused little interest (Sharon et al.,2004).As per the widely accepted definition by Goldstein, lectin is defined as "a carbohydrate-binding protein or glycoprotein of non-immune origin which agglutinates or precipitates glycoconjugates or both". Being glycoproteins, lectins bind to specific mono or oligosaccharides without altering the bound ligand and have the ability to agglutinate cells. Such glycoproteins are extensively found in plants, vertebrates, and invertebrates (Van Damme et al., 2008).These are most profoundly obtained from plants, being present in leaves, barks, seeds, tubers, rhizomes, roots, bulbs, depending on the plant species (Adenik et al., 2009; Wong et al., 2008; Audrey and Sharon., 2002; Thakur et al., 2007).Although initially isolated from plant seeds, lectins are now known to be ubiquitously distributed in nature (Sharon and Lis, 1989).Plant lectins are the most studied due to their highly specific interaction with carbohydrates and the biological effects based thereon. Phytolectins contribute significantly to biological research due to two main reasons. Firstly, plant lectins are a group of readily accessible carbohydrate binding proteins. Secondly, lectins isolated from plants are particularly suited for the examination, separation and purification of animal and human glycoconjuates because the latter compounds are the natural targets of most carbohydrate binding proteins present in plants. With the increasing recognition of their role in several cellular processes, there has been simultaneous progress in various areas of lectin biology and chemistry (Gabius *et al*., 2002; Goldstein, 2002; Hirabayashi, 2004)

In the past, lectins were classified in accordance with their carbohydrate specificities: mannose binding (Subramanyam et al., 2008), glucose binding (Naeem et al., 2007), galactose binding (Tsivileva et al., 2007) etc. As more lectins with diverse sugar-binding specificities were identified gradually, the old system was no longer feasible, and other classification systems were proposed. As per new classification system, animal lectins are classified into families of evolutionary related carbohydrate recognition domains (CRDs).Some major families include

calnexins (Ellgaard and Frickel, 2003), M-type, L-type, P-type, R-type, S-type (Vasta, 2012), I-type (Angata and Brinkman, 2002) and C-type (Zelensky and Gready, 2005) lectins. Based on the structure of lectins (Van Damme et al., 2008) introduced another classification system of lectins in which they were categorized into four diverse groups viz. hololectins, merolectins, chimerolectins and superlectins (Peumans and Van Damme, 1998). Likewise, plant lectins were also classified into 12 groups according to their structural and evolutionary relationships into different groups viz. legume lectins, jacalins, amaranthins (Van Damme et al., 2008; Lannoo and Van Damme, 2010).Although numerous plant lectins have been isolated, identified and characterized till date; yet there are numerous plant lectins left uncharacterized. Plant storage organs being rich in proteins are one of the best targets for identification and isolation of new plant lectins with greater yield, permitting extensive characterization of the lectins, assisting in the revelation of their biological potentials (e.g. anti-tumor activities) (Chan and Ng ,2013).

Lectins have found variety of applications nowadays, such as cell identification, separation, detection, isolation, structural studies of glycoproteins; investigation of carbohydrates on cells and subcellular organelles; histochemistry and cytochemistry; mapping of neuronal pathways;mitogenic stimulation of lymphocytes; purging of bone marrow for transplantation; selection of lectin-resistant mutants; studies of glycoprotein biosynthesis.(Sharon and lis,2004).The involvement of lectins in processes such as cell–cell and host–pathogen communications, serum-glycoprotein turnover and inborn immune responses are of particular relevance to tumour growth and metastatic spread. Several lines of evidence accumulated in the recent years implicate tumour cell lectins in cellular interactions viz. adhesion, cell growth, tumour cell differentiation, and metastasis (Singh & Sarathi, 2012). Lectins are also known to modify the cell cycle by inducing cell cycle seizure, apoptotic cell death and activating the caspase cascade. By virtue of changes in the production of various interleukins, they affect the immune system. There is also data to suggest that some lectins down-regulate telomerase activity and hence inhibit angiogenesis (Sharon and lis, 2004). A natural outcome of these studies has been the application of several lectins as therapeutic agents which favorably bind to cancer cell membranes or their receptors, thereby triggering cancer cell agglutination which translates into cytotoxicity, apoptosis, and inhibition of tumour growth (Sharon and lis, 2004). Although lectins

seem to have immense prospective as cancer markers and anticancer agents, numerous gaps in our knowledge relating to lectin biology still exist, necessitating more research on various fronts.

The peanut agglutinin (PNA) from peanut *(Arachis hypogaea* L.),is the first lectin to be fully studied in this plant.PNA has been reported to be highly specific for the tumor-associated T-antigenic disaccharide Gal (βl-3) GalNAc. Peanut agglutinin has been identified as a tetrameric protein with a molecular weight of 110 kDa. It particularly binds malignant cells; as a consequence, this lectin has been widely used as a probe for identifying malignant phenotypes in numerous tissues (Langkilde et al., 1992). The aim of the present investigation was to purify and characterize a lactose-binding lectin with similar properties from seeds of peanut. The present study was also intended to assess the *in vitro* anti-cancer properties of the isolated lectin.

3. REVIEW OF LITERATURE

Plant lectins are defined as those plant proteins which possess at least one non-catalytic domain which binds reversibly to a specific mono-or oligosaccharide .These are a class of carbohydrate-binding non-immune origin proteins ubiquitously distributed in a variety of plant species (Goldstein et al., 1980; Peumans et al., 2001). These can specifically recognize and bind to various sugar structures, thereby triggering several important cellular processes (Sharon, 1989; Sharon, 2007).Recent research in lectin biology has focused on the purification and characterization of hundreds of lectins with respect to their biological functions, biochemical properties and carbohydrate-binding specificities (Sumner, 1919; Van Damme et al., 1998). Classification of plant lectins is based on different criteria. Depending upon the overall structures of the mature plant lectins, they can be allocated into the following subdivisions ; 'merolectins', 'hololectins', 'chimerolectins', and 'superlectins' (Van Damme et al.,1998). Furthermore, plant lectins have also been classified according to their sugar-binding specificities into polyspecific (interact with more than one sugar) or monospecific (interact with a single sugar) (Vijayan et al., 1999; Barre et al., 2001).Analysis of available sequences has led to their classification into seven families of evolutionarily related proteins. Some lectins are yet to be allocated a group because they don't fit into any of the present classification systems and their sequence information is unavailable. Due to their ability to bind reversibly with specific carbohydrate structures, lectins have commonly been used as molecular tools in several disciplines of biology and medicine.

Lectins in plants are present mainly in seeds, cotyledons and kernels. Reported findings suggest that these lectins may have functions such as physiological regulation, defense against microorganism attack, storage protein, mitogenic stimulation, carbohydrate transport etc. within the plant. Plant lectins have effective biological activity and occur in foods like wheat, corn, tomato, banana peanut, kidney bean, soybean and many more. After consumption in diet, the ingested lectins show noticeable biological properties at biochemical and molecular level. Binding between lectins and surface cell molecules or internalization into cells involves a wide variety of signals that are important for cell regulation such as cell agglutination and/or

aggregation, induction of apoptosis or cell cycle arrest, down regulation of telomerase activity and inhibition of angiogenesis, increase of drug sensitivity of tumor cells, hence used as immunotoxins in cancer treatment.

3.1. LECTINS IN CANCER THERAPY

Tumor cells exhibit abnormal patterns of glycosylation in carbohydrates linked to ceramides and cell surface proteins (Hakomori, 1996; Gorelik et al., 2001; Hakomori, 1985). Membrane glycosylation alterations are present in all cancer cells and certain of them are well known as progression markers. During the different stages of the disease , each type of cancer presents differential alteration patterns (Nagata,2000).Glycosylation alterations that occur in cancer cells may comprise loss or changes in function of certain structures, presence of truncated structures or their precursors and, rarely the appearance of new structures. Tumor cells express carbohydrates that are either adhesion molecules as such or modulate adhesion receptor functions. The common changes include the increase of N-glycans and sialic acid content in the cell surface, the abnormal production of mucin, expression of Lewis X/A structures in glycosphingolipids, and the increased expression of galectins. Altogether, these changes link with the capacity of metastatic cancer cells and/or the increase in migration and their ability to evade the immune system (Hakomori, 1996; Chrispeels, 1999). In certain cases, membrane glycoproteins are also modified, thereby acting as oncogenic antigens. Recent findings implicate tumor cell lectins in cellular interactions such as adhesion, cell growth, tumor cell differentiation, and metastasis. The participation of lectins in progressions such as cell–cell and host–pathogen interactions, innate immune response and serum-glycoprotein turnover are of specific significance to tumor development and metastatic spread (Nagata, 2000). The awareness and understanding of the mode of interaction of lectins with cancer cells and their role in affecting the biology of the tumor will clarify the role of carbohydrates in the attainment of malignancy and consequently its inhibition (Nagata, 2000). Numerous studies have elucidated the ability of lectins to show preferential agglutination on cancer cells .It has been reported that a higher affinity is present between human cancer cells and lectins, as compared with healthy cells and the same lectins (Kuwahara et al., 2002). This is validated by the observed phenomenon of

selective binding of plant lectins, such as concanavalin A (ConA) and the wheat germ agglutinin (WGA) to tumor cells (Nagata,2000).Although, the link between membrane glycoproteins and lectins is weak one, but a stronger one is subsequently formed by multiple binding sites of a lot weak joints. Via this mechanism of interaction with altered glycosylation patterns present on cell surfaces of malignant cells, lectins can induce apoptosis, cytotoxicity, inhibition of tumor growth and can be used as diagnostic tools due to their ability of selective binding(González & Prisecaru,2005; Nishimura et al.,2004).

Different studies with various lectins have elucidated their anticancer properties *in vitro* and *in vivo*, via the process of preferential binding to cancer cell membranes or their receptors, thereby causing cytotoxicity, apoptosis, and inhibition of tumor growth (González & Prisecaru, 2005). Antitumor effect and anti-carcinogenic activity of lectins are due to different mechanisms. Lectins cause the induction of remission in certain tumors and may also have a direct anti-tumor cytotoxic effect. Certain lectins improve the antineoplastic effect of radiation and chemotherapy thereby showing a synergistic effect on cancer inhibition. They also promote restoration of normal growth in cancer cells and amplify the immunogenicity of tumor cells .Since they exhibit differential cytotoxic effect on malignant cells with respect to normal cells; they exhibit minimal risk of anti-tumor cytotoxic activity (Ruiz & Hernández, 2002). Lectins from different sources are known to inhibit cancer cells growth dependent on their concentration and in a differential manner (Pryme & Bardocz, 2002).The ability of lectins to modulate growth, differentiation, proliferation and apoptosis are mainly mediated by surface receptors (Abdullaev and Gonzalez, 1997).Comparative studies employing several lectins as antitumoral or cytotoxic agents have revealed differential effect depending on lectin source and cell line or cancer type. For example, experiments on the anti-cancer cytotoxic effect of lectins from common bean, soybean, with lymphoma cells revealed that each of the lectin had therapeutic effects, which was evidenced by inhibition of tumor growth and delayed tumor progression. Likewise, a study with five different lectins: PHA (*Phaseolus vulgaris*) ,GSA (*Griffonia simplicifolia*) ,Con-A (*Concavalina A*), WGA (*Triticum vulgare*) and PNA (*Arachis hypogea*) on three colon cancer cell lines (Lovo, HCT-15 and SW837) exhibited that growth was affected in different ways depending on the concentration and type of lectin tested which

subsequently led to the conclusion that these lectins had the property to inhibit growth of cancer cells in-vitro.Such findings have sparked a strong interest in research to use lectins as a treatment for tumor growth control .

Three major lectin families, namely, the legume lectins, the type II ribosome-inactivating proteins (RIPsII) and the GNA-related lectins, have been studied extensively due to their significant biological activities. The typical structural characteristics of these three lectin families (Waner et al., 1998; Ye et al., 2006; An et al., 2006; Van Damme et al., 2007) have been correlated with their biological activity. Plant lectins have been used as recognizing tools to differentiate malignant tumours from benign and the degree of glycosylation associated with metastasis. This has resulted in the adoption of several lectins such as mistletoe lectins for alternative cancer therapy (Schumacher et al., 2003).They are also indicated for the reduction of treatment-associated side-effects as adjuvant agents during chemotherapy and radiotherapy. Lately, many studies have additionally presented the anti-tumour actions of plant lectins on a range of malignant cells (De Mejía & Prisecaru, 2005; Choi et al., 2004).Numerous characteristic lectins such as MLs, Ricin and WGA have been reported to possess remarkable anti-tumour activities by inducing apoptosis in cancer cells. In addition, other lectins such as ConA and PCL have been reported to induce autophagy after internalization into tumour cells (Lei and Chang,2007; Cheng et al.,2009; Liu et al.,2009; Liu et al.,2009).The anti-tumour activity of plant lectins and their molecular mechanisms are well reported. Furthermore, the recent advances of pre-clinical and clinical studies of plant lectins have further revealed its potential as antineoplastic drugs in future cancer therapeutics.

3.2. PLANT LECTIN-INDUCED APOPTOSIS: THE MOLECULAR BASIS

Apoptosis, type I programmed cell death (PCD), is marked by condensation of the cytoplasm and nucleus, DNA fragmentation, cell contraction, dynamic membrane blebbing, chromatin merging in the nuclear periphery and phagocytosis (Michael,2000).Apoptosis is recognized as one of the most essential molecular mechanisms that can be regulated by numerous cellular signaling

pathways for tumour cell suicide (Cheng et al., 2009; Cheng et al., 2008).Cancer is closely associated with programmed cell death (PCD) or apoptosis, which is an evolutionary conserved process that plays a crucial role in metazoan development (Andrew,2008). Consequently, recognition of molecules or pathways of apoptosis required to suppress the tumorigenesis can lead to the adoption of apoptosis modulation as a new target for cancer therapy (Abdullaev and Mejia,1997).Modifying the vital molecular components of cell death machinery is an attractive approach for plant lectins (Pusztai et al.,2008). The following lectin families have been most intensely studied with regards to their apoptosis inducing abilities in cancer cells.

3.2.1. CHITIN-BINDING LECTINS

The anti-cancer mechanism of Wheat germ agglutinin (WGA), a typical chitin-binding lectin composed of hevein domains, has been researched extensively. It has been reported that WGA exerted strong inhibitory effects on the growth of the pancreatic tumour cells (Schwarz et al., 2009).WGA was highly toxic to human pancreatic carcinoma cells in vitro, with high levels of membrane binding to sialic acid residues and lectin internalization. Preceding studies on WGA reported its effect on lymphoma cell survival and found that it limited tumour growth (Chiara et al.,2009).Furthermore, the cell growth of human breast cancer cells in vitro (Zalatnai et al.,2000). was significantly affected by WGA.Moreover,it is reported that WGA induces chromatin condensation, nuclear fragmentation, and DNA discharge, which was consistent with apoptosis (Wang et al.,2000).

3.2.2. *Galanthus nivalis* AGGLUTININ (GNA)-RELATED LECTINS

Garlic lectin, one of the typical GNA-related lectins, isolated from garlic (*Allium sativum- L)* bulbs, is reported to possess cytotoxic effects in human tumour cells. This lectin possessed the ability to strongly inhibit DNA synthesis in human U937 and HL60 cells and induced apoptosis at a low concentration (Yuji et al., 2001).As a considerably consumed vegetable in daily life, some plants such as garlic have drawn more interests to the research of anti-tumour activities of GNA-related lectins. In recently reported findings, *P. cyrtonema* lectin (PCL) was shown to induce apoptosis in HeLa cells (Liu et al., 2008). Subsequent reports indicated towards the remarkable inhibitory effects of PCL and the other two GNA-related lectins, *O. japonicus* lectin

(OJL), and *L. noversa* lectin (LNL) on the growth of human breast adenocarcinoma MCF-7 cells. Furthermore, it has been reported that GNA-related lectins with additional mutated sites bind to extra types of sugar chains or carbohydrate containing receptors on the surface of tumour cells, which might activate more cell death signaling pathways (Tian et al.,2008).Lately, *Polygonatum odoratum* lectin (POL), a mannose-binding specific GNA-related lectin, has been reported to possess a remarkable anti-proliferative activity toward murine fiborsarcoma L929 cells by inducing apoptosis in a caspase-dependent manner. Such findings may provide new evidence for further exploring more apoptotic mechanisms of plant lectins in cancer research.

3.2.3. TYPE II RIBOSOME-INACTIVATING PROTEINS

3.2.3.1 Mistletoe (v. album) lectins

Mistletoe (V. album) lectins, classified as type II ribosome- inactivating proteins (RIPsII), have been at the receiving end of much attention owing to their unique anti-tumour mechanisms and therapeutical applications (Seifert et al.,2008). Mistletoe lectins typically comprise of a A-chain made up of three distinct individual domains and a B-chain containing two domains with similar configuration (Ganguly & Das,1994).It is postulated that the difference in the sugar-binding specificity of their B-chains may play a vital role in determining selective cytotoxicity for tumour cells by interacting with putative cell surface receptors.Furthemore, the cellular cytotoxicity of the lectins seems to require both the A- and B-chains (Siegle et al.,2001; Thies et al.,2005). Therefore, it has been postulated that the recognition and internalization of B-chain of the RIPsII via putative receptors might be a prerequisite for the A-chain to exert cytotoxic activity against cancer cells. Additionally, RIPsII are recognized to be exerting anti-tumour activity on cancer cells in different ways by targeting distinct stages of the apoptotic pathways.

3.2.3.2 Rice Bran Agglutinin (RAB) and Ricin

Rice bran agglutinin (RAB) has been reported to contribute to nuclear fragmentation, chromatin condensation, DNA release, and externalization of membrane phosphatidylserine in human monoblastic leukemia U937 cells (Miyoshi et al., 2001). A recent study has elucidated that ricin, a typical RIPII, induced cell death through the apoptotic pathways in L540 Hodgkin’s lymphoma

cells by upregulating of caspase-8 and subsequent downstream caspase 3/7 (Letizia et al.,2009). In addition to their remarkable anti-tumour activities, ricins are known to exert synergic effects with other anti-tumour agents in cancer cells. This could provide new molecular basis for their pharmaceutical applications in the near future.

3.2.4. LEGUME LECTINS

For several decades, legume lectins are one of the most comprehensively studied plant lectin families for their molecular basis of the protein– carbohydrate interactions (Damodaran et al., 2008). The main reason which has sparked the interests in this lectin family lay in their possible application as anti-tumour agents that could bind specific cancer cell surface glycoconjugates (Ueno et al., 2000). Concanavalin A (ConA), a typical legume lectin with a mannose/glucose-binding specificity, has been reported to induce apoptosis in murine macrophage PU5-1.8 cells via mitochondrial clustering and release of cytochrome c (Suen et al., 2000). The anti-tumor properties of concanavalin A have been further validated by recent studies showing that ConA induces apoptosis in human melanoma A375 cells in a caspase dependent pathway. Consequently, ConA caused mitochondrial transmembrane potential (MMP) collapse, cytochrome c release, activation of caspases and eventually triggering a mitochondria-mediated apoptosis (Liu et al., 2009). Other current reports have established that another legume lectin named *S. flavescens* lectin (SFL) can induce tumour cell death through a caspase-dependent apoptotic pathway. Its apoptotic mechanisms is hypothesized to be the death-receptor pathway (Liu et al.,2008).Another characteristic legume lectin worth mentioning in context of anti-tumour properties is purified from *Phaseolus coccineus L* with specificity towards sialic acid . *Phaseolus coccineus L.* seeds have shown to possess a remarkable anti-proliferative activity. This lectin has been reported to induce the caspase-dependent apoptosis in murine fibrosarcoma L929 cells. Another significant observation regarding anti-proliferative activity of this lectin was that its antineoplastic activity was decreased abruptly when the sialic acid-specific activity was completely inhibited. This indicates that this sugar-binding specificity might be the main reason sparking off the antineoplastic activity and apoptosis (Chen et al., 2008). To sum up, the mentioned discoveries of the legume lectins suggested that they might possess some similar biological activities and anti-tumour mechanisms that are closely correlated with their

corresponding molecular structures. These reported results would provide new clues for further exploring the anti-tumour mechanisms of the legume lectins.

3.3. PURIFICATION OF LECTINS BY AFFINITY CHROMATOGRAPHY

Affinity chromatography is a technique used to purify compounds, such as proteins, that have the ability to non-covalently and reversibly bind specific molecules, known as ligands. This method differs from the classical chromatography techniques in that the protein is purified on the basis of a unique biochemical property. In affinity chromatography, the ligand is covalently attached to a matrix, which needs to be chemically inert, porous, having a variety of functional groups suitable for coupling with diverse ligands. Lectins possess biochemical and binding properties which are very convenient for their purification by carbohydrate affinity chromatography. The binding of lectins to carbohydrates is noncovalent and reversible, involving hydrogen bonds, electrostatic hydrophobic, and van der Waals interactions and dipole attraction. Lectins do not react catalytically with carbohydrates, altering the ligand, lest the lectin is a subdomain of a modular protein that contains another catalytic (glycosidase) domain. Furthermore, lectins bind carbohydrates noncovalently and reversibly and at least the most widely used ligands, mono- and disaccharides, are usually bound relatively weakly, so that the lectin is readily released from an affinity column by competitive elution using specific free carbohydrates. Moreover, lectins and carbohydrates are both usually stable compounds, therefore elution techniques using extreme conditions of pH and/or ionic strength can also be applied to release a lectin from the carbohydrate affinity column. Carbohydrate affinity chromatography is a simple, one-step method for purifying lectins. However, in some cases additional separation techniques have to be used subsequently to purify the lectin to homogeneity, such as ion-exchange chromatography and/or gel filtration.

3.4. LEGUME LECTINS: A BRIEF OVERVIEW

Lectins derived from leguminous plants are most extensively studied (Sharon et al., 1990).These glycoproteins may comprise up to 3% of the weight of a mature seed. Research highlighting protein and gene sequencing has revealed that most of the leguminous lectins are made up of either two or four protomers of about 30 kDa.Although all legume lectins are related at the molecular level but show a significant variation in carbohydrate binding specificity (Table-1) which certainly contributed to the success of legume lectins as therapeutic tools. Significance to legume lectins are given since they are abundant in many crop plants and their association in the symbiosis between legumes and the nitrogen fixing bacterium *Rhizobium* is well documented (Diaz et al., 1989). Lectins are generally widely distributed amongst leguminous plants (Toms, 1971). Together, the legume lectins exhibit numerous similar properties like metal ion-binding, and all of them are tetrameric glycoproteins possessing identical or nearly identical subunits (Liener., 1976; Lis., 1972). Subject to their common source and upon their retaining similar physical, chemical, and biological properties, many of the legume lectins are homolog (Foriers, 1977).Moreover, the in vitro function of this group of proteins is well documented and the molecular basis of this interaction has been studied with a variety of biophysical techniques. The legume lectins form a large family of homologous proteins. Pair wise sequence identities of not lower than 35% are observed in the 50 legume lectin sequences. Recent studies have indicated that legume lectin homologues may be present in the animal kingdom also (Fiedler, 1994).

Lectin	Sugar specificity
Arachis hypogaea (peanut)	Galactose
. *Bandeiraea simplicifolia*	Galactose
Bauhinia purpurea alba	N-acetyl galactosamine
Dolichos biflorus	N-acetyl galactosamine
Glycine max (Soybean)	N-acetyl galactosamine
Lotus tetragonolobus	Fucose
Phaseolus lunatus (Lima bean)	N-acetyl galactosamine
Phaseolus vulgaris (PHA-E; Kidney bean)	Galactose
Ricinus communis (Castor bean)	Galactose
Sophora japonica (Pagoda tree)	N-acetyl galactosamine
Ulex europeus (Gorse)	Fucose
Vigna radiata (Mung bean)	Galactose
Wisteria floribunda	N-acetyl galactosamine
Concavalin ensiformis (Jack bean)	Glucose/mannose
Lens culinaris (Lentil bean)	Glucose/mannose
Pisum sativum (Garden pea)	Glucose/mannose

Table-1: Legume lectins and their Sugar Specificity

3.5. PEANUT AGGLUTININ

The peanut agglutinin (PNA), which is the first lectin to be fully studied in this plant is specific for the tumor-associated T-antigenic disaccharide Gal (βl-3) GalNAc.It is a homotetrameric protein with a molecular weight of 110 kDa. PNA has been reported to bind malignant cells of breasts. Hence, this lectin has been extensively used as a probe for detecting malignant phenotypes in several tissues (Langkilde et al., 1992). PNA agglutinates neuraminidase-treated human erythrocytes (Sun et al., 2008). The agglutinin specificity of PNA for terminal *β*-D-galactosyl residues has enabled its purification by Sepharose 6B column chromatography. Sepharose-coupled on *α*-aminocaproyl-β-D-galactopyranosylamine by affinity chromatography, lactosaminylAE-P-150, agar polyacrylamide beads and lactosyl- sepharose (Gray and Baues., 1977; Sutoh et al., 1977; Fish et al., 1978).

The lectin from peanut (*Arachis hypogaea*),with specificity for the tumor-associated T-antigenic disaccharide Gal(B1-3)GalNac,is like Con A,a tetrameric protein with M.W 110,000(Lotan et al.,1975).Each subunit in the protein is 236 amino acid residues long and is homologous to subunits in other legume lectins (Young et al.,1991). The molecule dissociates at low pH into dimers which bind sugar with the same stoichiometry as the tetramer (one binding site per protomer) .The association constant for the dimers is one order of magnitude lower than that for the tetramer (Fish et al., 1978; Decastel et al., 1985). This property of dissociating at low pH is a characterisitic property exhibited by Con A (Senear & Teller,1981; Senear & Teller,1981) and other distinguished tetrameric proteins such as hemoglobin (Fanelli et al.,1964; Wyman,1964).However, at physiological pH the molecule is entirely tetrameric, with no evidence of association-dissociation.The isoforms of lectins show differences in thermal stability. At pH 7.4, the temperature of maximal stability of the peanut agglutinin tetramer has been calculated to be -33 °C (Reddy et al., 1999).PNA was the second tetrameric lectin whose structure was determined. In 1982, its crystallization was reported but the refined structure at 2.2A° was only studied recently (Banerjee, 1994).

Despite the fact that each subunit in PNA has the same characteristic tertiary fold that is usually

found in other legume lectins, the structure of PNA displays an uncommon quaternary arrangement of subunits (Banerjee et al., 1994).Despite the fact that it has some homology with other known legume lectins, its structure could only be solved by replacing with isomorphs. The tetramer of peanut lectins usually comprises of two GS-IV type dimers depicted in (Fig- 1). GS-IV type dimers containing two monomers each associate in a similar fashion but not identical to the canonical dimer. However, no continuous 12-stranded β-sheet is formed along the dimer interface. The two N-terminal strands of the two monomers are intercalated by a series of 6 water bridges. Since the internal symmetry of PNA does not form a closed point group, its tetramer is thus different among all homo tetrameric proteins. As PNA is not a glycoprotein, its unique quaternary structure is attributed to intrinsic properties of the protein itself.

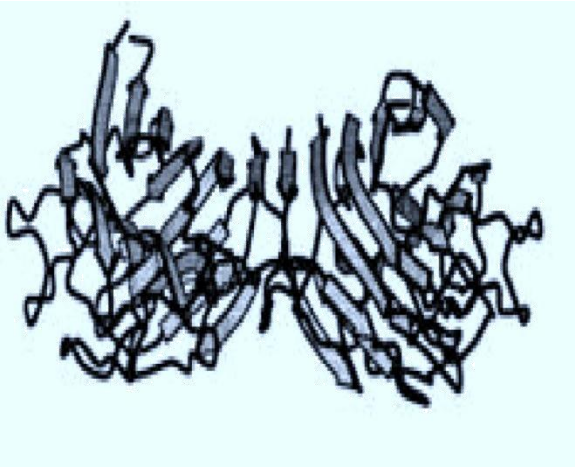


Fig 1.1 Canonical Dimer

Fig 1.2 GS4 Dimer

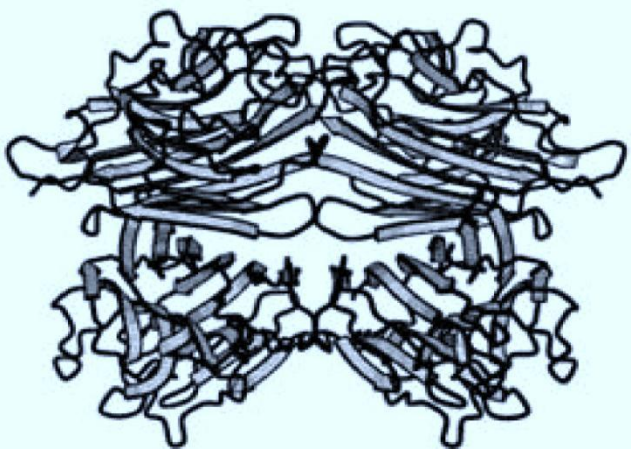


Fig 1.3 PNA Tetramer

3.5.1 PNA: ROLE IN CANCER DIAGNOSIS AND THERAPY

Peanut agglutinin plays a vital role for the detection of mature and immature thymocytes preceding bone marrow transplantation (Reisner et al., 1979). It also helps in the detection of malignant tumors). Peanut (*Arachis hypogaea*) agglutinin generally recognizes the Thomsen-Friedenreich antigen, a T-antigenic determinant (Galb1–3GalNAc) which is chemically well studied tumor-associated antigen having link to malignancy in man (Irimura. et al., 1975; Salunke. et al., 1985). These structures are usually found as *O*-linked glycans by poorly differentiated cells and tumor cells, but these cells are absent or modified in normal cells. The tumor-associated Thomsen-Friedenreich binding antigen from peanut does not associate with either GalNAc or other galactose derivatives with bulky C-2 substituent (Swamy et al., 1991; Lotan et al., 1975; Reisner et al.1979; Younget al., 1981). By flow cytometric method, PNA can be used to study keratinocyte sub populations .The difference between basal and supra basal keratinocytes due to PNA binding is observed early in the epidermal development with the onset of stratification (Watt et al., 1989). Immunoelectron microscopy shows that the glycoproteins are mostly found on the cell surface microvilli and absent in desmosomal junctions. PNA-binding glycoproteins play a crucial role in cell-cell adhesion, but so far there has not been any data that directly support the idea (Morrison et al., 1988; Watt et al., 1989). CD44, a multifunctional polymorphic integral membrane glycoprotein share a number of features of the keratinocyte PNA-binding glycoprotein, that is a receptor for hyaluronan (Lesley et al., 1993b).A strong correlation has been found between PNA binding to the primary carcinoma cells and PNA binding to their lymph node metastases. Detection of early colorectal cancer imaged with peanut agglutinin-immobilized fluorescent nanospheres having surface poly(N-vinylacetamide) chains has also been reported (Sakuma et al.,2010).Furthermore, cultured breast cancer cells express on their surface glycoproteins which are recognized by the peanut lectin (PNA).It has been reported that the proliferation of these cells (ZR-75.1 and 734-B) was inhibited by PNA.The combination of PNA with either retinoic acid or 4-hydroxy-tamoxifen resulted in an additive amplification of the antiproliferative activity. Synergistic effect of PNA is evident by findings suggesting the enhanced growth-inhibitory action of interferon-gamma in combination with PNA.

OBJECTIVES OF THE STUDY

1. **Isolation of Peanut Agglutinin from peanut (*Arachis hypogaea)* seeds.**
2. **Characterization of the isolated lectin**
3. **Elucidation of the *in vitro* anti-cancer properties of peanut agglutinin**

4. MATERIALS AND METHODS

4.1. Materials

Dried peanuts (*Arachis hypogaea*) were purchased from local grocery at Rourkela, Odisha, India and were used for all the experiments in this study. Human blood type AB+ and O+ were obtained from healthy donors with the assistance of pathologists at CWS hospital, Rourkela, Odisha, India.

4.2. Chemicals and consumables

Sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), glycine, copper sulphate ($CuSO_4$),DAPI, trypan blue, crystal violet, potassium sodium tartarate ($KNaC_4H_4O_6$) ,Acrylamide, bisacrylamide, Ammonium persulphate (APS), Sodium dodecyl sulphate (SDS), N,N,N',N'-tetramethylenediamine (TEMED), ethanol, bovine serum albumin (BSA), neuraminidase from *Clostridium perfringens (C. welchii),*were purchased from Sigma Aldrich, India. folin-ciocalteau phenol reagent, potassium dihydrogen phosphate (KH_2PO_4), potassium hydrogen phosphate (K_2HPO_4) was purchased from S.D. fine Chem. Ltd., Mumbai. DMEM, MEM, FBS, acetic acid, bromophenol blue, agarose were purchased from Himedia, Mumbai. glycerol was purchased from Rankem Pvt Ltd.,pre stained molecular weight marker was purchased from Bio-Rad, India.,methanol, silver nitrate, sodium thiosulphate were purchased from Nice chemicals Pvt. Ltd. India.

96 well microtitre plate, T25 and T75 cell culture plates were purchased from Tarson,India.

4.3. Preparation of Lactamyl Sepharose affinity matrix

METHODOLOGY:

About 8g of sepharose 4B was washed by PBS in filtration unit and stored in 40ºC in alcohol.

Epoxy activation of Sepharose 4B:

About 8g of sepharose 4B was suspended in 12 ml of distilled water and mixed well through pipette. This was followed by addition of 5.2ml of 2M NaOH. Next, about 1.3ml of

epichlorohydrin was added so that the final concentration of the various components were 30% (v/v) sepharose, 5% epichlorohydrin, 0.4M NaOH.Then, the suspension was incubated at 40ºC for 2 hours with continuous shaking. It was then transferred to a glass filter funnel and the gel was washed with 500ml of distilled water.

Preparation of Amino Sepharose 4B:

Epoxy activated sepharose 4B was suspended in 1.5 volume of concentrated ammonium solution (12ml).The suspension was then incubated at 40ºC for 90 mins. Then, it was again transferred to glass filter funnel and the gel was washed with distilled water.

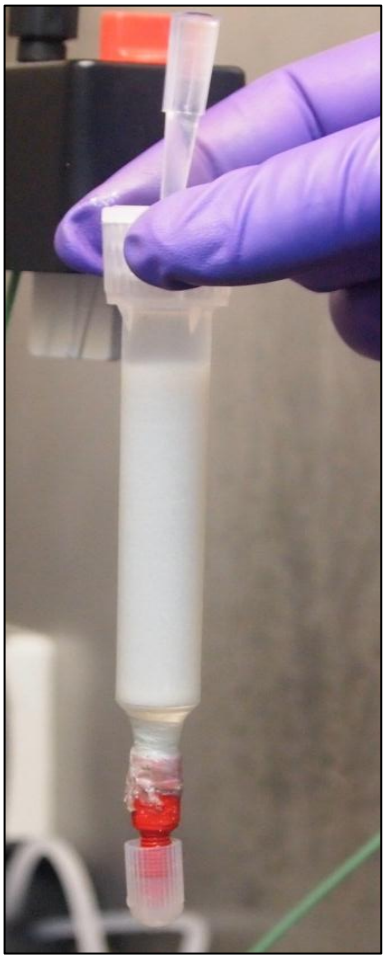


Fig 2: Lactamyl sepharose column

Coupling of lactose with Amino Sepharose 4B:

8g of the dried amino sepharose 4B was then suspended in 6ml of 0.2M K_2HPO_4 buffer containing 208mg lactose and 102mg of $NaCNBH_3$.The suspension was then incubated at room temperature for 10 days with occasional shaking. Following 10 days of incubation, the free amino groups which remained in the gel were acetylated by adding 2ml of acetic anhydride. The suspension was then incubated at room temperature for 1 hour. The lactamyl sepharose 4B thus obtained was subsequently washed with distilled water, 0.1 M NaOH, distilled water and 10 mM PBS. Then it was stored in distilled water with traces of sodium azide at 4ºC.

4.4. Extraction and Purification of Lectin

100g of peanut was taken and crushed into small pieces by mortar pestle. The crushed peanuts were then soaked overnight in 250 ml of 10mM PBS at pH 7.2. The soaked peanuts were grinded well by grinder using the PBS in which it was soaked. Then, the grinded mixture was centrifuged at 8000 rpm and 4ºC temperature for 20min. Supernatant acquired after centrifugation was then used for purification of lectin. Proteins in the supernatant were precipitated by salting out method using ammonium sulphate (20-60% saturation). After 60% cut off, the precipitate or the pellet was resuspended and dialyzed for 1 day against distilled H_2O followed by dialysis for 3days against 10mM PBS (pH 7.2) till all the salts were released. Then it was applied to affinity chromatography on the prepared lactamyl-sepharose column which was pre equilibrated with the same buffer. Affinity chromatography was carried out in a FPLC (Fast Protein Liquid Chromatography) unit (GE Aktaprime plus). The dialysed sample was loaded onto the column connected to the FPLC unit. Flow rate was maintained at 0.5 ml/min and unbound materials, which did not bind to the column (indicated by the 1st peak)(Fig 2), were washed from the column .Elution of all unbound proteins was indicated by the subsidence of the 1st peak. After the graph showed a trend of zero absorbance for some time, 5ml of 0.5M lactose was injected into the column .The onset of a 2nd peak (Fig 2) indicated the elution of our protein of interest and this fraction which showed absorbance peak at 280nm was collected and dialyzed extensively against 10mM PBS (pH-7.4) at 4ºC overnight to remove the lactose. The dialysed

fraction was then lyophilized and the lyophilized powder was further used for characterization of the isolated lectin and assessment of its anti-cancer properties.

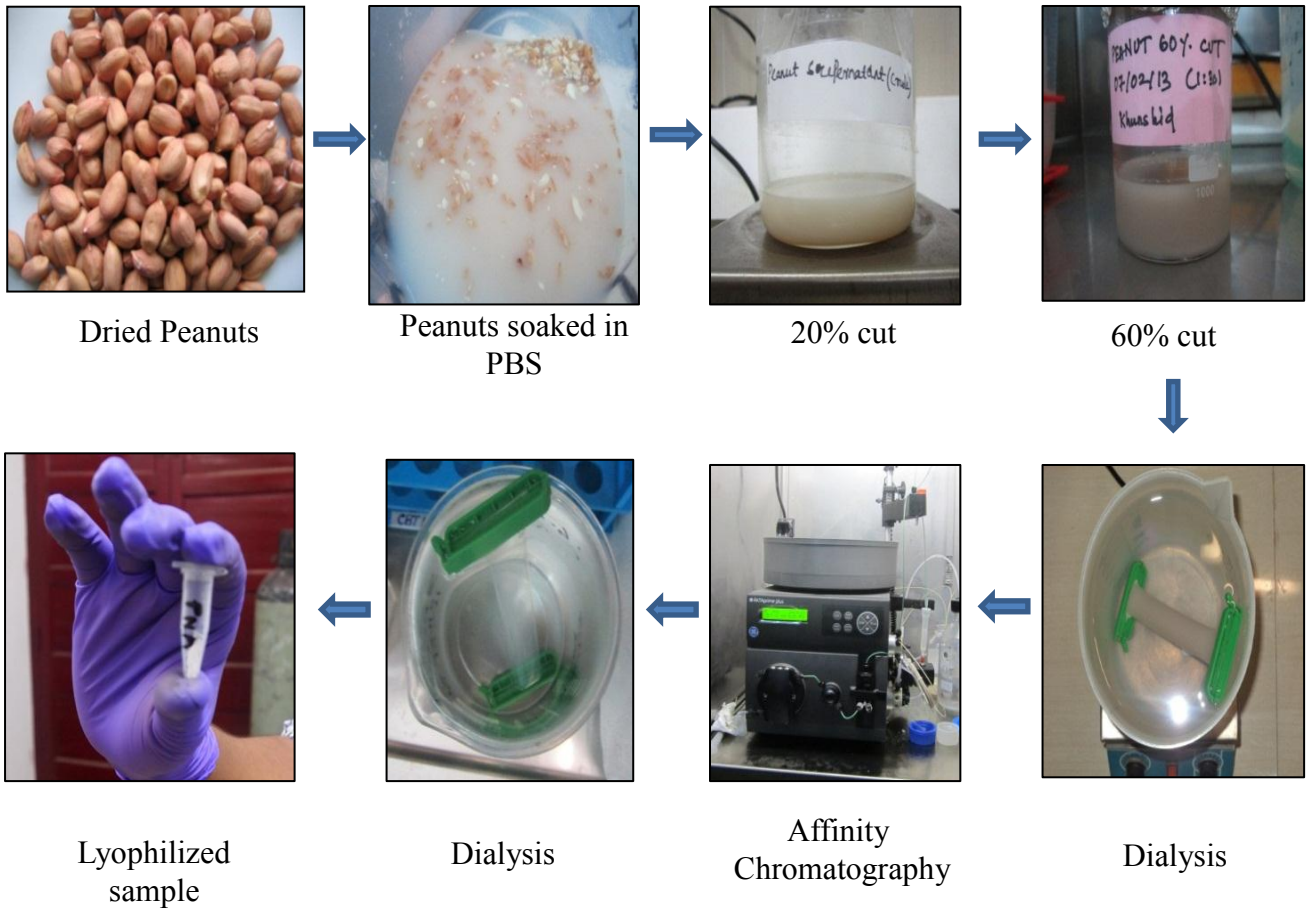


Fig 3: Steps followed for isolation and purification of the lectin

4.5. Cell Culture

Hela, Hep-2, HaCat cell lines were obtained from NCCS, Pune and ATCC. Hela, Hep-2 and HaCat cell lines were maintained in MEM and DMEM supplemented with 10%FBS and penstrep. The cell lines were maintained at 95% humidity in a CO_2 incubator and the cultures were allowed to grow till 80-90% confluency before drug treatment.

4.6. Determination of concentration of protein by Lowry method

PRINCIPLE:

The Lowry assay is based on the reaction of cupric ions with peptide bonds under alkaline conditions (the Biuret test). Protein samples are mixed with an alkaline solution containing copper sulphate (Cu^{2+} ions) which reacts with peptide bonds to produce Cu^{+} ions – strong reducing agents. Afterwards, Folin-Ciocalteau reagent is added where upon the Cu^{+} ions in the solution react with the Mo (VI) ions to form molybdenum blue – a complex of Mo (IV) and Mo (V) ions. The blue color of the dye can then be measured at an absorbance of 600 nm. The color produced is a direct reflection of protein concentration and, with the use of standards, can facilitate determination of protein concentration.

PROCEDURE:

The concentration of crude, 20% cut off, 60% cut off, and affinity fraction were measured by Lowry et al. using bovine serum albumin as the standard protein

Reagents required:

1. BSA stock solution (1 mg/ml)
2. Analytical Reagents

    A- 50 ml of 20% sodium carbonate mixed with 50ml of 0.1N NaOH solution (0.4gm in 100ml of distilled water)

    B- 10 ml of 1.56% CuSO4 solution made with 10ml of 2.37% sodium potassium tartarate solution. Prepare analytical reagents by mixing 2ml of B with 100 ml of A

3. Folin's reagent-ciocalteau reagent solution (1N) dilute commercial reagent (2N) with as equal volume of water on the day of use (2ml distilled water and 2 ml of reagent)

Different dilutions of BSA stock solutions were prepared.0.2 ml of protein sample was taken to which 2ml of alkaline $CuSO_4$, mixed, then kept for 10mins incubation. Next, 0.2 ml of Folin's reagent was added to it and incubated for 30 mins. in dark. Then O.D reading was taken at 600nm using Perkin Elmer 2030 reader .The graph was plotted for determining the unknown

concentration of the protein of interest taking absorbance in the abscissa and concentration in the ordinate.

4.7. Haemagglutination Assays

PRINCIPLE:

Lectins are named agglutinins because of their characteristic property of agglutinating cells such as erythrocytes by binding to specific receptors present on the surface of these cells.Hence,the HA is of vital importance in characterizing the isolated protein as a lectin. Haemagglutination is visible macroscopically and is the basis of haemagglutination assays to detect the presence of lectins. The haemagglutination assay can be used to quantify the amount of lectins present in suspensions. This is done by carrying out serial dilutions of the lectin suspension in a microwell plate and then testing to determine an end point. The result thus obtained can be used to determine the quantity of agglutinin in the suspension which is expressed as a HA titre.

PROCEDURE:

Haemagglutination assay was carried out using AB+ and O+ blood groups .The HA was performed in neuraminidase-treated erythrocytes and neuraminidase-untreated erythrocytes. First, HA was performed for neuraminidase-untreated erythrocytes .For this, about 1ml of human blood was collected in presence of anticoagulant from the CWS hospital. After three washes with 10mM PBS,the erythrocytes were suspended in the same solution to a content of 2% (v/v).The lyophilized protein obtained previously was used to prepare a 1 mg/ml stock solution. This stock solution was then serially diluted in 96 well "U" bottom micro titer plate with 10 mM PBS. .Three wells were taken as negative control i.e. containing PBS only. The erythrocyte suspension was then added in each well in equal amount and kept for incubation at room temperature for observing agglutination. This method was followed for both AB+ and O+ blood groups.

NEURAMINIDASE TREATMENT

Neuraminidase treatment of erythrocytes was carried out using neuraminidase from *Clostridium perfringens* (Type V) lyophilized powder(6.1 units/mg solid).First, 2% (v/v) erythrocyte

suspension was prepared using the above-mentioned procedure and neuraminidase treatment was done by adding 15 µl of neuraminidase/ml of 2% (v/v) erythrocyte suspension followed by incubation for 1 hr at 37°C.Neuraminidase treated cells were again washed thrice with 10mM PBS and 2%(v/v) erythrocyte suspension of the neuraminidase-treated erythrocytes was prepared. Different dilutions of the protein stock solution were prepared in 96 well "U" bottom micro titer plate as mentioned above. The erythrocyte suspension was then added in each well in equal amount and kept for incubation at room temperature for observing agglutination. This method was followed for both AB+ and O+ blood groups.

4.8. Electrophoresis

The purity of the lectin was determined by SDS-PAGE and Native PAGE using 8% total concentration polyacrylamide as the resolving gel and 5% polyacrylamide as the stacking gel. The molecular weight of peanut lectin was determined by SDS–PAGE under reducing condition using beta mercaptoethanol and SDS adopting the method of Sambrook and Russel, 2001.The molecular weight of the native protein was determined by native PAGE adopting the method of Sambrook and Russel, 2001.Silver staining method was used for viewing the protein bands in each case.

4.9. MTT assay for assessing the effect of PNA on HeLa cell viability

PRINCIPLE:

Conventionally, cell growth estimation is done by counting viable cells after staining with a vital dye. In the mitochondria of living cells, Yellow MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) is reduced to a purple product, formazan .The absorbance of the formed colored solution can subsequently be computed by measuring at a certain wavelength (typically between 500 and 600 nm) by a spectrophotometer. The absorption maxima is dependent on the solvent used. Only active mitochondrial reductase enzymes could catalyze this reduction .Consequently, conversion can be directly related to the number of viable (living) cells. The effectiveness of the agent in instigating death of cells can be inferred by

comparing the amount of purple formazan produced by cells treated with that agent with the amount of formazan produced by untreated control cells, via the production of a dose-response curve.

PROCEDURE:

In this study, MTT assay was carried out to assess the cytotoxic effect of PNA in varying concentration on HeLA, HaCat and Hep-2 cell lines. The cells were reharvested in T25 culture flask and trypsinised when they reached 80-90% confluency. The cells were then seeded in 96 well plate. After 24 hrs, the cells were treated with varying concentration of PNA with 6 replicates in each concentration and kept for 72 hrs incubation. MTT was then added and kept for about 4 hrs. Next, DMSO was added to dissolve the purple formazan and OD reading was taken at 562nm using Perkin Elmer 2030 reader.

4.10. Trypan blue exclusion assay for determination of viable cell number

PRINCIPLE:

This dye exclusion test is used to determine the number of viable cells present in a cell suspension. The principle behind such tests is that live cells possessing intact cell membranes would exclude certain dyes viz. trypan blue, propidium or Eosin, whereas dead cells do not. For this test, a cell suspension is just mixed with dye and then visually scrutinized to ascertain whether cells take up or exclude dye. A viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm due to the inability to exclude the trypan blue dye.

PROCEDURE:

Trypan blue exclusion assay was carried out using varying concentrations of PNA on HeLa cells using the Invitrogen protocol .Cell count for viable cells was carried out using Olympus 1X71 microscope.

4.11. DAPI staining for study of apoptosis by morphological changes in nuclei of PNA treated HeLa cells

PRINCIPLE:

DAPI or 4', 6-diamidino-2-phenylindole is a fluorescent stain that binds strongly to A-T rich regions in DNA. Cells undergoing apoptosis display typical features, viz. reduction in cell-size, membrane blebbing, chromatin condensation and nuclear fragmentation. Nuclear shrinkage, chromatin condensation, and formation of apoptotic bodies can simply be detected under fluorescence microscopy following appropriate staining of nuclei with DNA-specific fluorochromes such as DAPI .DAPI can pass through an intact cell membrane therefore it can be used to stain both live and fixed cells.However,it efficiency in passing through membrane is less in live cells and therefore the effectiveness of the stain is lower.

PROCEDURE:

Preseeded HeLa cells were treated with varying concentrations of PNA followed by incubation for 24 hrs. Then, after discarding the old media, the cells were treated with 4% paraformaldehyde for 30 mins. Then washing with PBST was done followed by washing with PBS twice.Next,DAPI (1X) was added to the cells and incubated in dark for 5 mins.Then,the cells were washed again with PBS twice and observation was carried out under Olympus 1X71 microscope.

4.12. DNA Laddering assay for study of PNA-induced DNA fragmentation in HeLa cells

PRINCIPLE:

DNA laddering is a distinctive feature of DNA degraded by caspase-activated DNase (CAD), which is a key event during apoptosis. CAD cuts genomic DNA at internucleosomal linker regions, thereby producing DNA fragments that are multiples of 180–185 base-pairs in length. The cleaved fragments are separated by agarose gel electrophoresis and subsequent visualization

be done by ethidium bromide staining, which results in a characteristic "ladder" pattern.

PROCEDURE:

Preseeded HeLa cells were treated with varying concentrations of PNA and after 24 hrs incubation, PNA induced DNA fragmentation was assessed by the DNA laddering assay protocol adopted from Suman et al.,2011.

4.13. Clonogenic Assay for assessing the long-term cytotoxicity of PNA on HeLa cells

PRINCIPLE:

Clonogenic assay or colony formation assay is an in vitro cell survival assay based on the ability of a single cell to develop into a colony defined to be consisting of at least 50 cells. This assay essentially tests each and every cell in the population for its capacity to undergo limitless division. Clonogenic assay can be used to determine the long-term effectiveness of certain cytotoxic agents on tumour cells that can grow in culture.

PROCEDURE:

HeLa cells were seeded in 6-well plates and following 24 hrs incubation, PNA treatment was done with varying concentration of the drug. After rinsing with the fresh medium, PNA-treated HeLa cells were allowed to grow for 14 days to form colonies. Thereafter, staining was done with crystal violet and colony counting was done macroscopically.

5. RESULTS

5.1. AFFINITY CHROMATOGRAPHY PEAKS

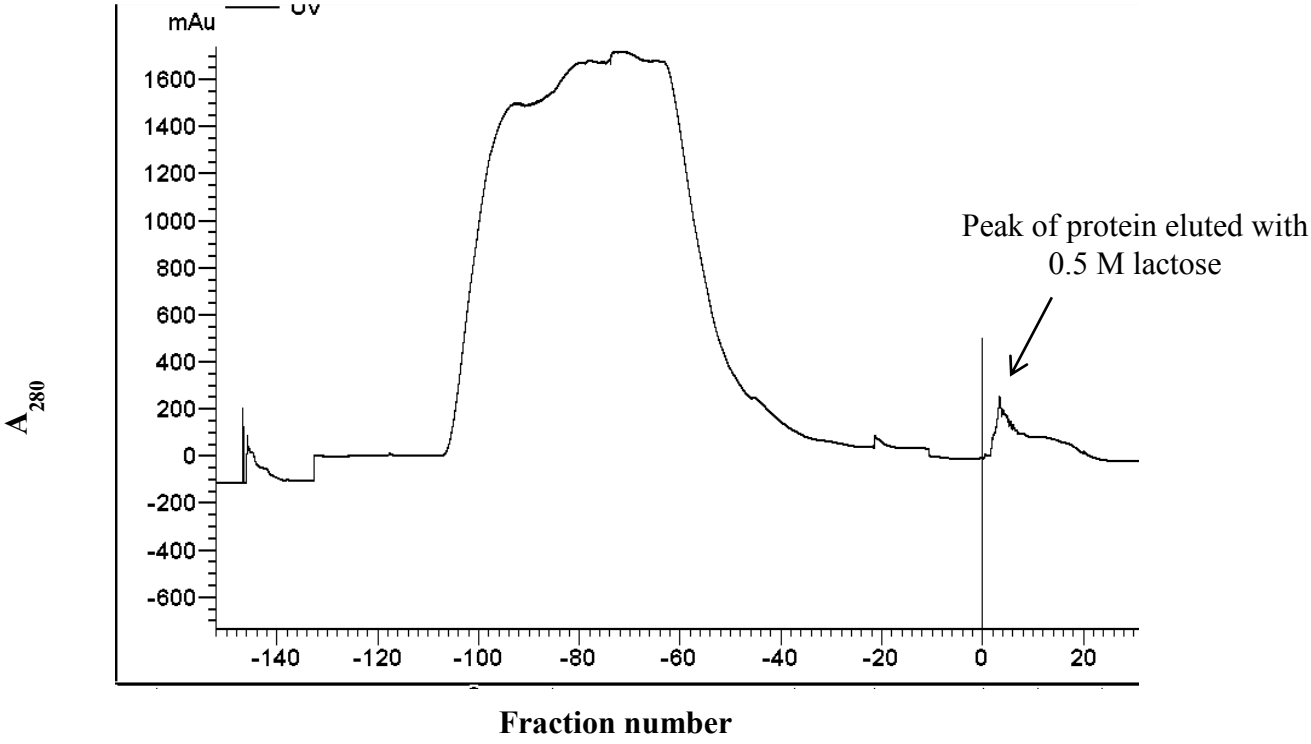


Fig 4: Affinity chromatography peaks

INFERENCE

The affinity chromatography peaks (Fig 4) obtained while isolating the lectin using lactamyl sepharose column clearly indicates that after the elution of unbound proteins with PBS ,our lectin of interest i.e. a galactose-binding lectin was eluted from the column by injecting 0.5 M lactose.

5.2. ESTIMATION OF PROTEIN CONCENTRATION

Estimation of protein concentration for crude, 20%, 60% and affinity fractions was carried out by Lowry method and the following results were obtained (Table 2).

Sample	Volume (ml)	Concentration (mg/ml)	Total protein content (mg)
Crude	84	19.91	1672.44
20% cut	60	13.95	837.00
60% cut	45	6.68	300.60
Affinity	30.75	0.40	12.30

Table 2: Estimation of protein concentration by Lowry method

INFERENCE

The final protein concentration in the elute obtained after affinity chromatography was found out to be 12.30 mg by Lowry method. The affinity purified fraction after dialysis followed by lyophilization yielded 12 mg protein (approx.) in lyophilized powder form which was used for further characterization assays and tests for determining anti-cancer properties of the isolated lectin.

5.3. HAEMAGLUTTINATION ASSAY

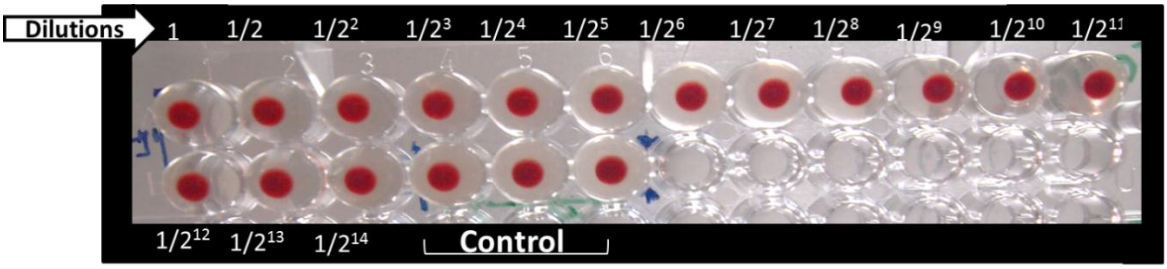


Fig 5.1: HA of untreated O+ blood group

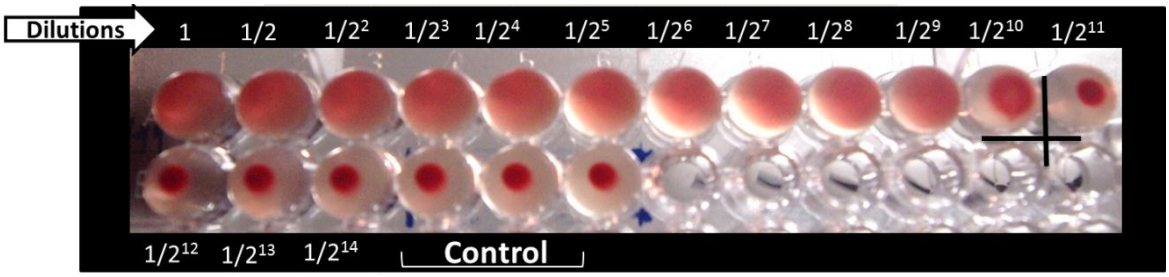


Fig 5.2 : HA of neuraminidase-treated O+ blood group

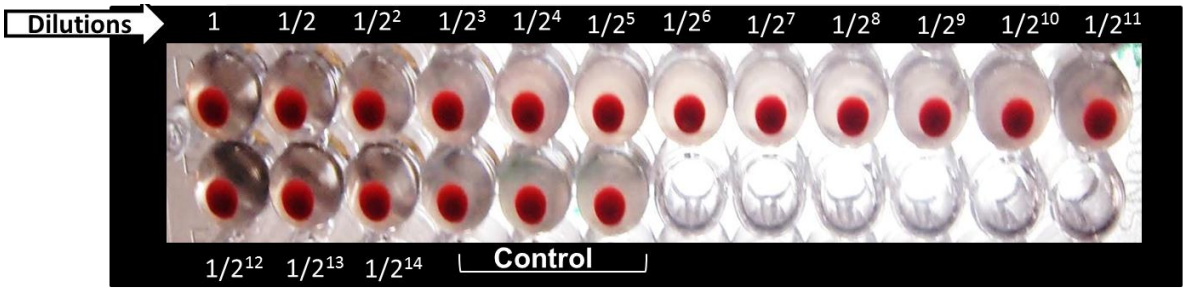


Fig 5.3: HA of untreated AB+ blood group

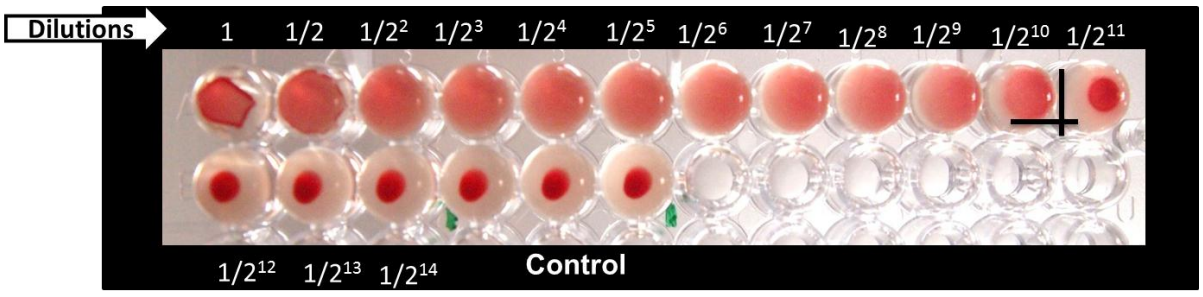


Fig 5.4: HA of neuraminidase-treated AB+ blood group

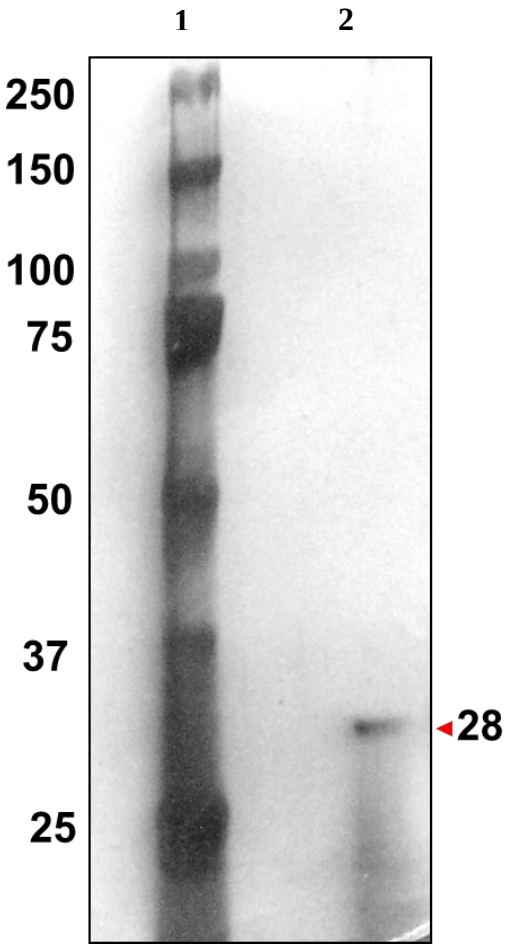
Blood group	Titer value
O+	1024
AB+	1024

Table 3: Estimation of titer values for agglutination of different blood groups with PNA

INFERENCE

To characterize the protein, haemagglutination assay was performed by using human erythrocyte suspension (AB and O).It was observed that the lectin could not agglutinate untreated human erythrocytes implying that the lectin was unable to bind to the erythrocytes However, after the treatment of the cells with neuraminidase there is a great enhancement in the binding of the lectin which resulted in the agglutination of erythrocytes. This property of binding specifically to neuraminidase-treated erythrocytes is known to be displayed by Peanut agglutinin. The isolated lectin was found to agglutinate AB+ and O+ human erythrocytes equally showing a titer value of 1024 in each case.

5.4. SDS PAGE



Lane 1 – Standard protein marker (Biorad)

Lane 2 –PNA (1mg/ml)

Fig 6: Silver stained SDS-PAGE (8%) Electrophoretogram

*(All MW represented in kDa)

INFERENCE

A single prominent band at 28 kDa was observed (Fig 6) .Studies on Structural conformation of PNA have revealed it to be a homotetramer of 110 kDa composed of four identical subunits of approximately 27kD each. Thus, SDS PAGE result is in agreement to the established findings regarding homotetrameric structure of PNA.Furthermore, a single prominent band indicates the purity of the isolated lectin.

5.5. NATIVE PAGE

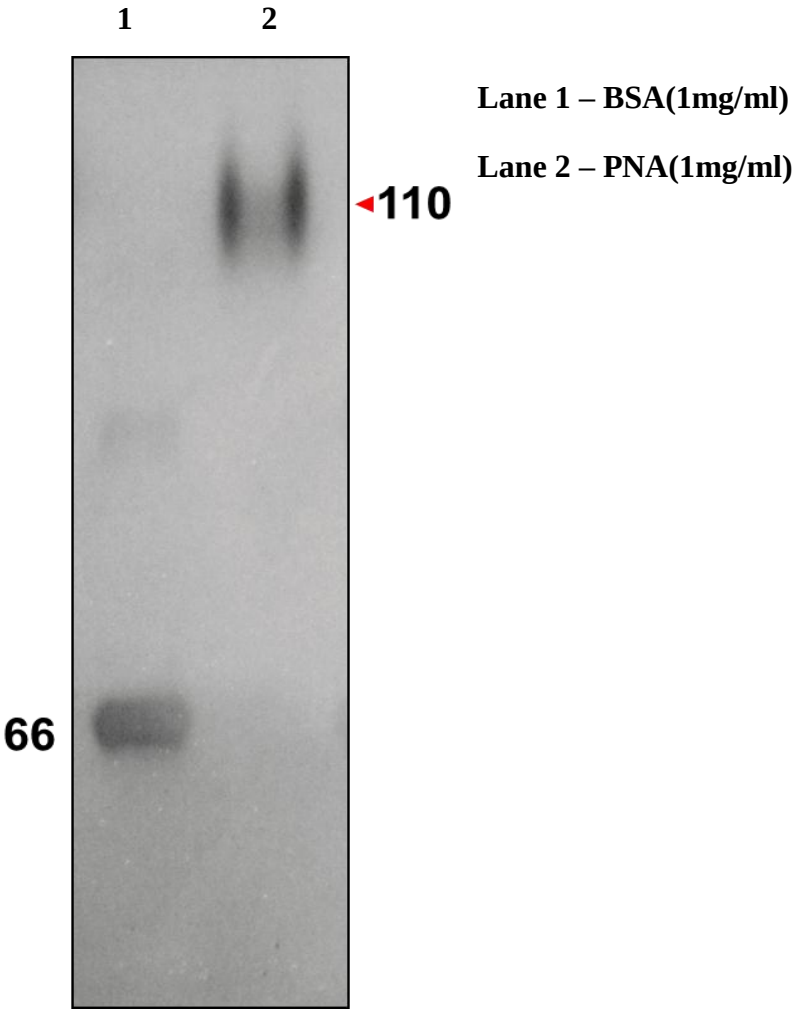


Fig 7: Silver stained Native-PAGE (8%) Electrophoretogram

***(All MW represented in kDa)**

INFERENCE

A single prominent band at 110 kDa was observed(Fig 7).Studies on structural conformation of PNA have revealed it to be a homotetramer of 110 kDa Thus, native PAGE result is in agreement to the established findings regarding homotetrameric structure of PNA.Furthermore,a single prominent band indicates the purity of the isolated lectin.

5.6. MTT ASSAY

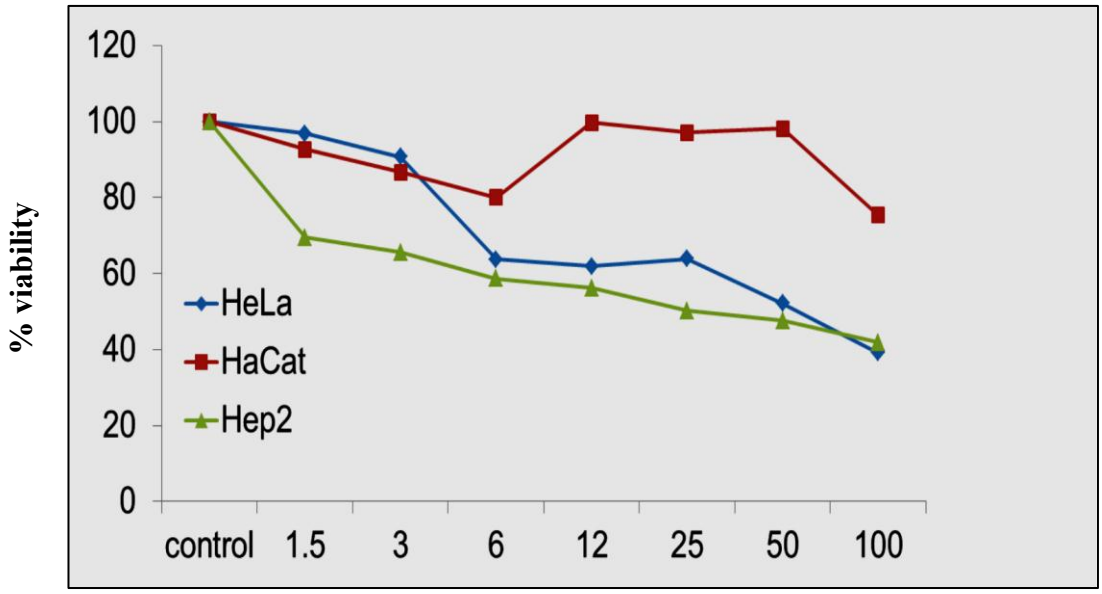


Fig 8: Graph showing cell viability of PNA treated HeLa cells in MTT assay

Cell line	IC_{50} (µg/ml)
HeLa	11.25
Hep-2	9.67
HaCat	>100

Table 4: IC_{50} (µg/ml) for different cell lines treated with PNA

INFERENCE

PNA was found to reduce the viability of HeLa, Hep-2 cell lines at indicated concentrations with six replicates in each concentration (Fig 8).Moreover, the IC_{50} value for the normal human cell line i.e. HaCat is significantly higher than that of cancer cell lines viz. Hep-2 & HeLa (Table

3).This indicates that PNA is selectively cytotoxic to cancer cell lines at lower concentrations and a significantly higher dose is required to inhibit the proliferation of normal human cells in vitro.

5.7. TRYPAN BLUE EXCLUSION ASSAY

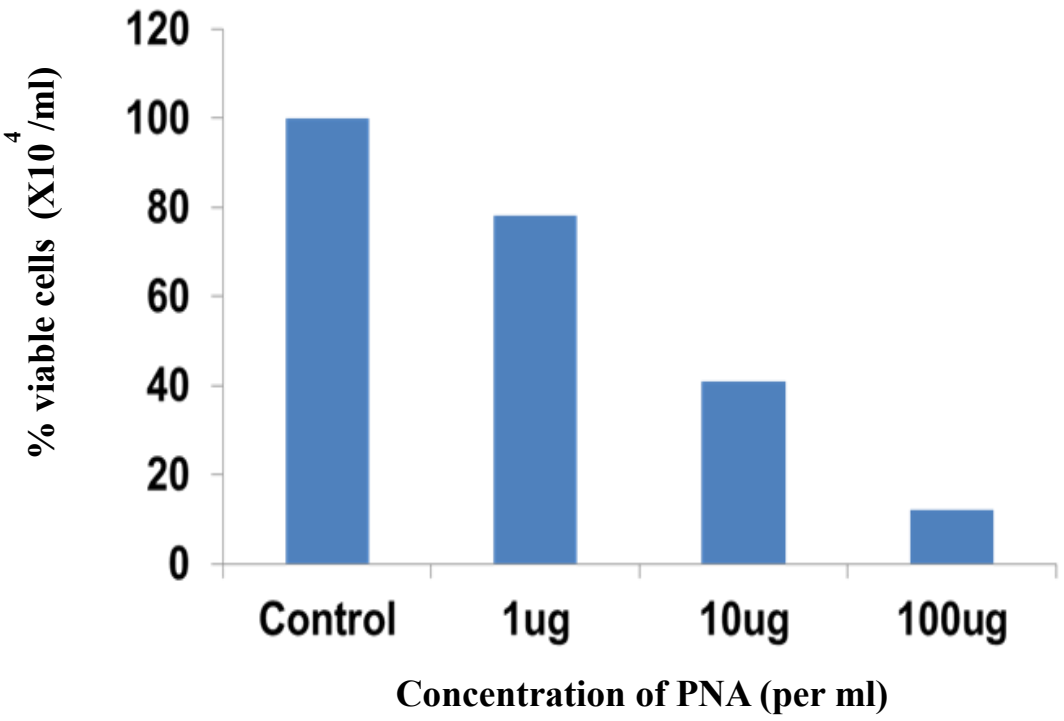


Fig 9: Graph showing % of viable HeLa cells post PNA treatment

INFERENCE

Marked increase of cell death with increment of PNA dose in HeLa cells was observed (Fig 9).Thus; this dye exclusion method further validated the cytotoxic effect of PNA on HeLa cells in a dose-dependent manner.

5.8. DAPI STAINING

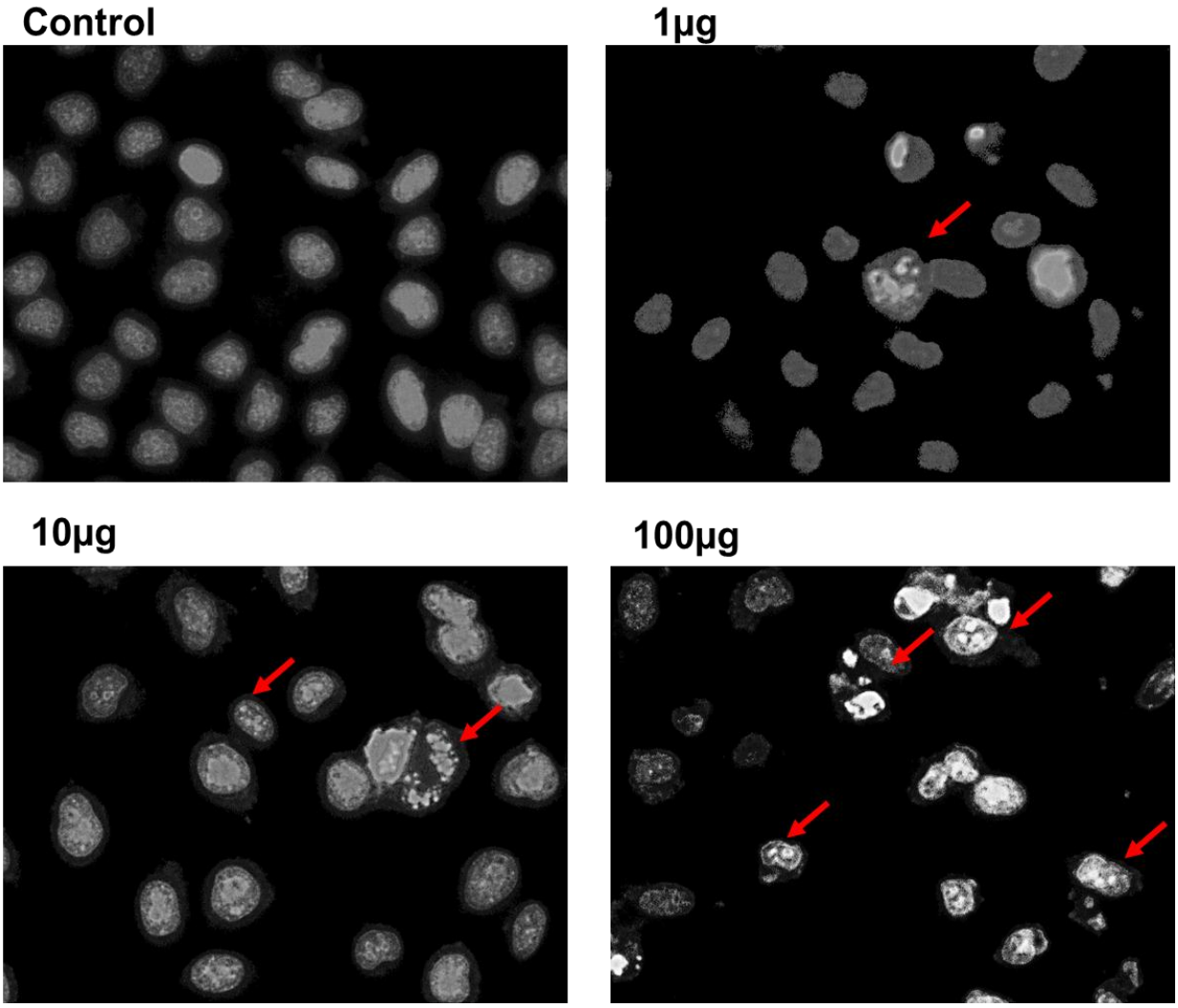


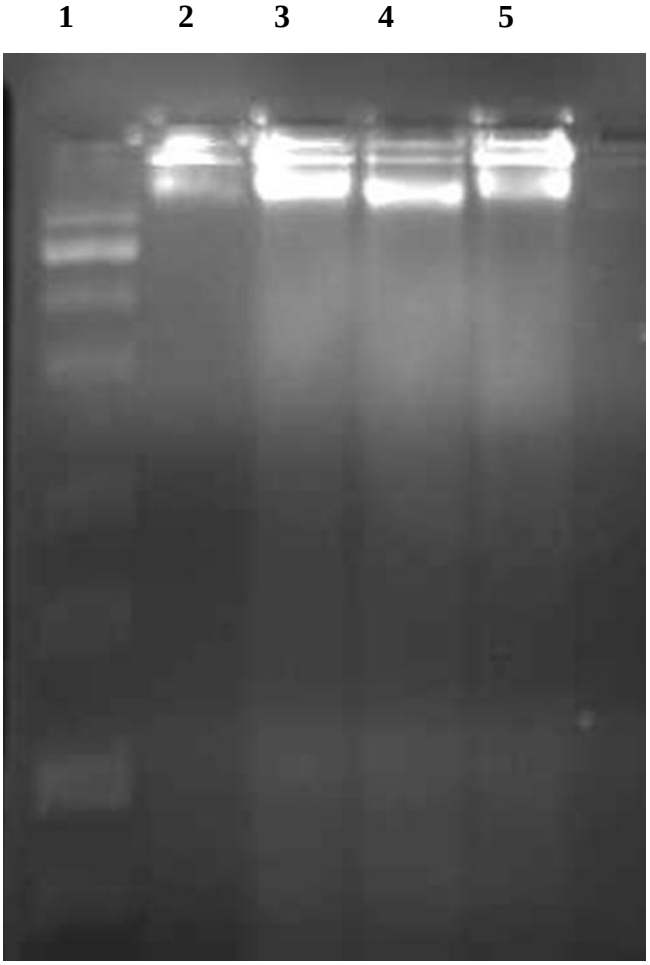
Fig 10: Observation of Morphological changes in nuclei of PNA treated HeLa cells by DAPI staining

***PNA concentration indicates in µg/ml**

INFERENCE

It was inferred that chromatin condensation and nuclear fragmentation in PNA-treated Hela cells increased in a dose-dependent manner which might indicate to possible apoptosis.

5.9. DNA LADDERING ASSAY



Lane 1-Marker
Lane 2 -PBS
Lane 3 -PNA 1 µg/ml
Lane 4 - PNA 10 µg/ml
Lane 5 - PNA 100 µg/ml

Fig 11 :EtBr stained 1.5% agarose gel showing DNA laddering in PNA treated HeLa cells

INFERENCE

Typical DNA laddering was observed after 24 h exposure when the HeLa cells were exposed to various concentrations of PNA (Fig 11) indicating the occurrence of DNA fragmentation at these concentrations.

5.10. CLONOGENIC ASSAY

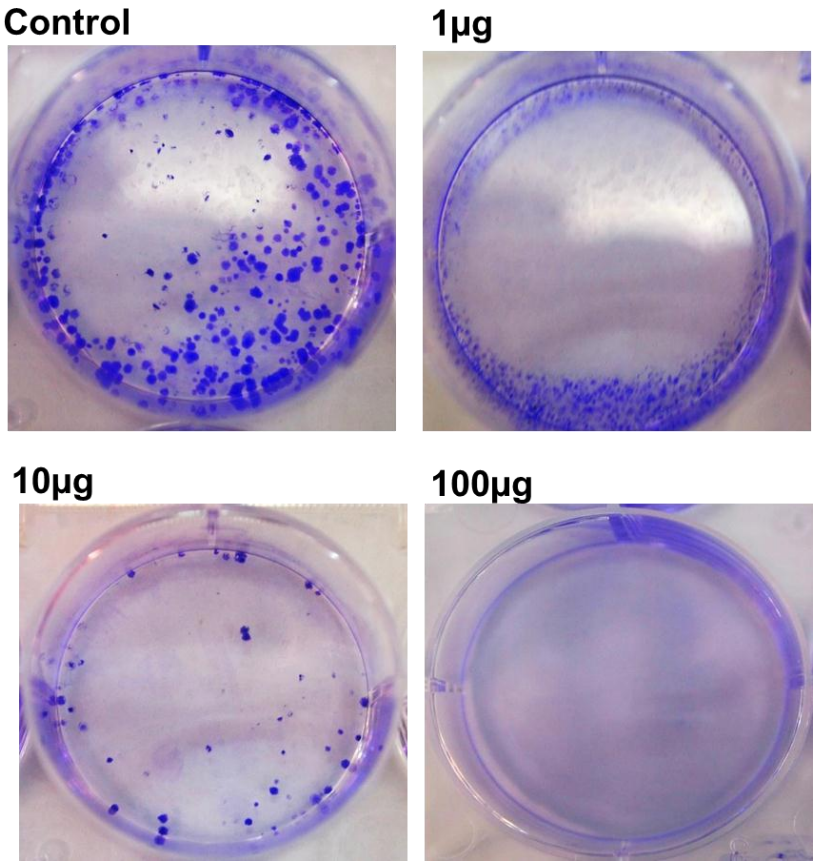


Fig 12.1: Crystal violet stained HeLa cell colonies

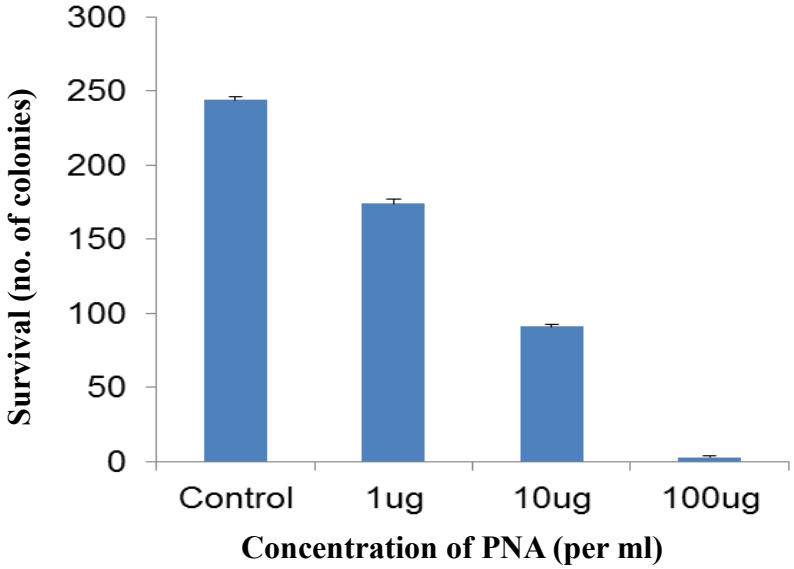


Fig 12.2 : Graph showing survival rate of of Hela colonies with increasing PNA concentration

INFERENCE

The colony forming assay was performed to examine the effects of PNA on colony forming ability of HeLa cells .A dose dependent colony forming inhibition effect was observed(Fig 12.1 ,12.2).

6. DISCUSSION

Plant lectins are described as a class of carbohydrate-binding non-immune origin proteins ubiquitously distributed in a variety of plant species .Plant lectins are renowned for their property to specifically recognize and bind to various sugar structures which triggers several important cellular processes. In the past few years, numerous plant lectins have been purified and characterized in details with respect to their carbohydrate-binding specificities, biochemical properties and biological functions. Lectins possess biochemical and binding properties which are very convenient for their purification by carbohydrate affinity chromatography. The binding of lectins to carbohydrates is non-covalent and reversible process, involving hydrogen bonds, electrostatic, hydrophobic, and van der Waals interactions and dipole attraction. The lectin peanut agglutinin (PNA) binds to D-galactosyl residues and to sequences of D-galactose and N-acetyl galactosamine (Novogrodsky et al., 1975; Pereira et al., 1976).Studies have indicated that the immunocompetent thymic-derived (T) cells do not bind PNA since the surface galactosyl residues are covered with sialic acid groups (Novogrodsky et al., 1975; Reisner et al., 1979) which appear to be mounted on the glycoproteins during the course of cortical thymocyte differentiation (Abel and Grey, 1975; Haessli and Pink, 1980).This phenomenon was also observed in our study as the PNA didn't have the capacity to agglutinate human erythrocytes .This was because glycoconjugates for PNA present on erythrocyte surfaces are masked by Neu5Ac,which is the major type of the sialic acid found in human erythrocytes, which account for 30% of the total sialic acid respectively . PNA was unable to combine NeuAc on the surface of erythrocytes. However, post neuraminidase treatment which removes the NeuAc on the surface of the erythrocytes, PNA could agglutinate these human erythrocytes. Moreover,the property of lectins to specifically recognize and bind to various sugar structures have prompted recent studies to focus on utilizing lectins as anti-cancer agents exhibiting specific cytotoxicity towards tumor cells. Tumor cells exhibit abnormal patterns of glycosylation in carbohydrates linked to ceramides and cell surface proteins (Hakomori, 1996; Gorelik et al., 2001; Hakomori, 1985). Membrane glycosylation alterations are present in all cancer cells and certain of them are well known as progression markers. During the different stages of the disease, each type of cancer presents differential alteration patterns (Nagata, 2000). Numerous studies have elucidated the ability of lectins to show preferential agglutination on cancer cells. It has been reported that a higher affinity is present between human cancer cells and lectins, as compared with healthy cells

and the same lectins (Kuwahara et al., 2002). This is validated by the observed phenomenon of selective binding of plant lectins, such as Concanavalin A (ConA) and the wheat germ agglutinin (WGA) to tumor cells (Nagata, 2000).The present study with Peanut Agglutinin has also validated the above phenomenon. In this study, PNA, a legume seed lectin that is specific for D-galactose residues at non-reducing terminal positions of glycoconjugates, was successfully isolated from *Arachis hypogaea* seeds using a lactamyl-sepharose column. In this study,HeLa (cervical cancer cell line) and Hep-2(oral cancer cell line) were chosen for studying anti-cancer cytotoxic effects of the isolated lectin because oral cancer and cervical cancer are the major culprits in cancer-related deaths in India. The isolated PNA was shown to exhibit marked cytotoxicity towards the proliferation of human cancer cell lines viz. HeLa and Hep-2 as indicated by MTT assay,trypan blue assay and clonogenic assay. Recent studies have suggested that modifying the vital molecular components of cell death machinery is an attractive approach for plant lectins (Pusztai et al., 2008). Lectin families such as Type II ribosome-inactivating proteins, chitin binding lectins, GNA related lectins, legume lectins have been most intensely studied with regards to their apoptosis inducing abilities in cancer cells.In this study, the ability of the isolated lectin i.e. PNA to induce DNA fragmentation in human cervical cancer cell line (HeLa) was indicated by DAPI staining and DNA laddering assay which may indicate to possible apoptosis. Further studies on cell-death inducing pathways (apoptotic and autophagic) of PNA are required to elucidate the underlying mechanism of lectin-induced cell death in cancer cells which will provide extra impetus for researching the prospect of PNA in therapeutics for specific cancer types in the near future.

7. CONCLUSION

The D-galactose binding lectin PNA was successfully isolated and purified from the seed of peanut (*Arachis hypogaea*) using a lactamyl sepharose column .The D-galactose binding Peanut lectin was eluted by injecting 0.5 M lactose . The purity of these proteins was confirmed by SDS-PAGE and native PAGE which confirmed the isolation of PNA which is a homotetrameric protein of M.W 110 kDa .The lectin was shown to agglutinate neuraminidase-treated human erythrocytes of AB+ and O+ blood groups equally showing a titer value of 1024 in each.However,PNA could not agglutinate untreated human erythrocytes. The anti-cancer property of PNA was elucidated by cell viability assays viz. MTT assay, trypan blue assay and clonogenic assay. The isolated PNA showed marked cytotoxicity towards human cancer cell lines viz. HeLa and Hep-2 in a dose-dependent manner. The specific cytotoxicity of PNA towards cancer cell lines was validated by comparing the significant difference in the IC_{50} values of PNA for human cancer line (HeLa, Hep-2) and normal human cell line (HaCat) used in this study. The isolated lectin was also found to induce DNA fragmentation in treated HeLa cells in a dose dependent manner, as indicated by DAPI staining and DNA laddering.

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